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THE SHAPING OF THE ORNAMENTED FERTILIZATION MEMBRANE OF *COMANTHUS JAPONICA* (ECHINODERMATA: CRINOIDEA)

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The fertilization membrane of echinoderms typically has an outer surface that appears smooth by light microscopy. However, some crinoid echinoderms are exceptional, since they have a fertilization membrane covered with conspicuous protuberances (Mortensen, 1920a, 1920b, 1937, 1938; Dan and Dan, 1941). Although the protuberances were originally thought to be spines, more recent work has shown them to be ridges (Holland and Jespersen, 1973). These ridges are produced and shaped by the female germinal cell acting without the aid of ovarian follicle cells; in this respect, the crinoid fertilization membrane differs fundamentally from egg envelopes bearing surface processes produced and shaped by the follicle cells, as in some teleosts (Wourms and Sheldon, 1976) and chitons (Richter, 1976). According to Mortensen (1920a), the main function of the ridges on the crinoid fertilization membrane is to reduce the sinking rate and facilitate drift dispersal of the planktonic eggs and embryos.

In their light microscopic study of *Comanthus japonica*, an unstalked crinoid, Dan and Dan (1941) showed that surface topography is transferred from the oocyte to the fertilization membrane. Light microscopy, however, could not convincingly demonstrate how this transfer is accomplished. Therefore, in the present investigation, the formation of the fertilization membrane of *Comanthus japonica* is described by electron microscopy. The results leave little doubt that oocyte topography acts as a first template, shaping a pattern of jelly on the surface of the unfertilized egg; this jelly, in turn, acts as a second template, shaping the pattern of ridges on the outside of the fertilization membrane. The morphogenetic role of the jelly is corroborated in the present study by data showing that a smooth surfaced fertilization membrane can be produced if the jelly is first removed from the living egg.

Materials and Methods

Specimens of *Comanthus japonica* were collected in Koaziro Bay, Kanagawa Prefecture, Japan, from a few days to a few hours before spawning; the date and even the hour of spawning can usually be predicted in advance (Dan and Kubota, 1960). Animals were held individually in dishes of running sea water at the nearby Misaki Marine Biological Station. Oocytes were obtained by dissecting ovaries, and ripe eggs were obtained from females spawning in the laboratory. For insemination, one drop of undiluted sperm from a testis of a male was mixed rapidly with several thousand eggs in 100 ml of sea water at $22 \pm 1°$ C.

For transmission electron microscopy (TEM), oocytes and eggs were fixed for 90 min at room temperature in 3% glutaraldehyde in 0.1 M Sorensen's phosphate

buffer (pH 7.3) with 0.45 M sucrose. Specimens were rinsed at room temperature in the same buffer with 0.45 M sucrose before post-fixation for 60 min on ice in 1% osmic acid in the same buffer with 0.45 M sucrose. This procedure, which is illustrated by Figures 4, 11, 13, and 21, gave good cytoplasmic fixation, but preserved the egg jelly poorly or not at all. The jelly was preserved for TEM, with less than optimal fixation of cell membranes, by a modification of the method of Behnke and Zelander (1970). Oocytes and eggs were fixed for 90 min at room temperature in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.5) with 1% alcian blue 8GN (B408AX440) and 0.35 M NaCl; the NaCl was added immediately before use. Specimens were rinsed at room temperature in the same buffer with 0.5 M NaCl, but the postfixation of Behnke and Zelander (1970) was omitted, since it removed the jelly. This TEM fixation with alcian blue is illustrated by Figures 3, 7, 8, 14, 16, and 17. All TEM specimens were rapidly dehydrated in an ethanol series, transferred to propylene oxide and embedded in Epon. Contrast of silver sections was enhanced with uranyl acetate and lead citrate.

For scanning electron microscopy (SEM), oocytes and eggs were fixed several days at room temperature in a solution of 2% glutaraldehyde in dilute (77%) sea water; this fixative was approximately isotonic with full strength sea water. The procedure removed the jelly, as illustrated by Figure 10. The jelly was preserved for SEM by the modified method of Behnke and Zelander (1970), which has been described in the preceding paragraph. This SEM fixation with alcian blue is illustrated by Figures 1, 2, 5, 6, 9, 12, and 15. All SEM specimens were rapidly dehydrated in an ethanol series, transferred through Freon 113 and dried by the Freon critical point method. The dried oocytes and eggs were mounted on stubs with double-stick tape (Scotch Brand), rotary coated with a mixture of gold and palladium (60:40) and viewed in a Cambridge S4 scanning electron microscope.

Jelly was removed from living eggs by a 30-min incubation in calcium-free sea water prepared according to Harvey (1956, p. 156). Such eggs, even if returned to normal sea water, did not respond to insemination; however, they did activate after treatment in ionophore by the method of Steinhardt and Epel (1974). For activation, 5 μl of a 5 mM solution of ionophore A23187 (Eli Lilly Co.) in dimethylsulfoxide was mixed rapidly with several hundred eggs in 5 ml of sea water (with or without calcium) at 22 $\pm$ 1° C. Appropriate controls of dimethylsulfoxide without ionophore never resulted in egg activation.

Results

The dented oocyte

Throughout the last week of oogenesis in *Comanthus,* the oocyte surface (Fig. 1) is dented with several hundred pits, each about 10 μ across by 10 μ deep. Every pit contains a conspicuous mass of extracellular jelly. This jelly appears to be produced by the oocyte and is rich in nonsulfated acid mucopolysaccharides (Holland, Grimmer and Kubota, 1975). Each mass of jelly will be termed a jelly clump and should not be confused with the copious but diffuse mucus (probably of epidermal origin), which briefly surrounds the eggs after spawning (Holland and Grimmer, 1975). A jelly clump, as seen at relatively low magnification, consists of interconnected strands and globules (Figs. 2 and 3). Some of the strands are

closely apposed to the oocyte's plasma membrane at the bottom of the pit (Fig. 3, single arrow) and appear to hold the jelly clump in place. At higher magnification, the jelly has a finely granular consistency.

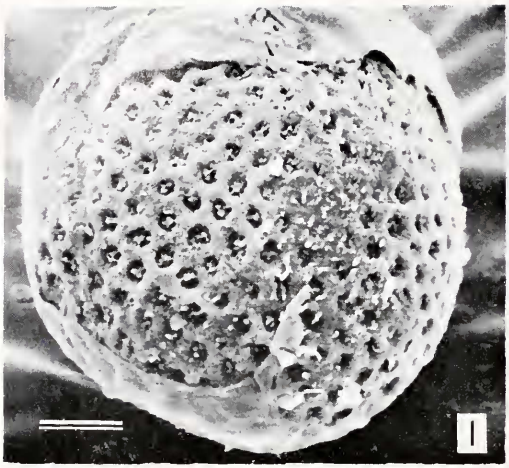
The plasma membrane of the oocyte is folded into ridges called microplicae (as defined by Andrews, 1976). The microplicae, which are too small to be visible in Figure 1, occur on all parts of the oocyte surface, whether undented (Fig. 2) or dented (as determined by serial sectioning). In cross section, each microplica is about 0.5 μ high and just over 0.1 μ wide and is capped with a thin layer of extracellular material several hundred Ångstroms thick (Fig. 4, arrow). The cortical cytoplasm of the oocyte includes cortical granules (Fig. 3, twin arrow), which are membrane-bound organelles about 1 to 3 μ diameter. The finely granular contents of each cortical granule are divisible into irregular dense patches and a less dense matrix (Fig. 4).

The unfertilized egg

Oocytes differentiate into eggs prior to spawning. The unfertilized egg (Figs. 5–7) no longer has the pitted surface that characterized the oocyte. However, the jelly clumps remain distributed over the egg surface in the same pattern they had on the oocyte (Figs. 5 and 6). Each clump probably indicates the position of a former pit on the oocyte surface. In comparison to the jelly clumps of the living egg (Fig. 18), those of the fixed egg are somewhat shrunken (Fig. 5). As seen at higher magnification the egg jelly has a substructure suggestive of closely packed tubules (Fig. 8). Each tubule has a dense wall and a lucent core. Tubule diameters are roughly 250 Å, and tubule lengths may reach at least several thousand Ångstroms; there is no apparent order in the arrangement of the individual tubules. The jelly clumps seem held in place on the egg surface (Fig. 7) by close association with the outside of a vitelline coat. The vitelline coat (Figs. 8, 11, and 21) is a moderately dense layer of extracellular material about 100 to 150 Å thick, which closely follows the plasma membrane around the circumference of the egg. Most of the vitelline coat suddenly appears while the oocyte is differentiating into the egg a few hours before spawning. It is not known whether the vitelline coat incorporates any of the extracellular material that capped the oocyte's microplicae. Instead of microplicae, the egg surface bears scattered microvilli up to 0.5 μ long (as demonstrated by serial sections). Beneath the plasma membrane of the egg, cortical granules are located in the cortical cytoplasm (Fig. 7).

The cortical reaction and fertilization membrane formation

Exocytosis of cortical granules begins about 45 sec after insemination of the *Comanthus* egg at the presumed point of sperm entry; the cortical reaction then spreads over the egg surface to the opposite pole in about 60 sec (Dan and Dan, 1941). On the egg in Figure 9, the cortical reaction started at the far left and progressed roughly halfway over the surface by the instant of fixation. In surface view, the leading edge of the reaction is obscured by the jelly clumps in Figure 9, but can be seen on eggs from which the jelly has been removed (Fig. 10). Just behind the leading edge, the newly formed fertilization membrane is ridgeless; then, some 25 μ behind the leading edge, the erection of the ridges begins.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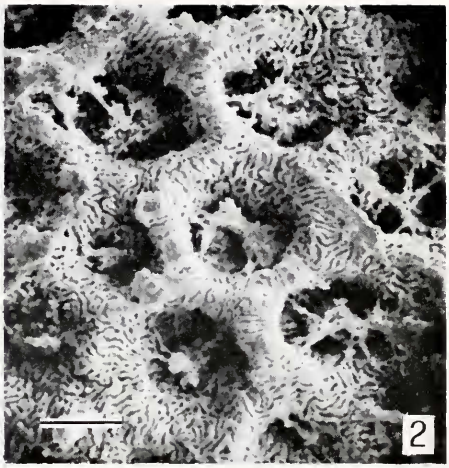
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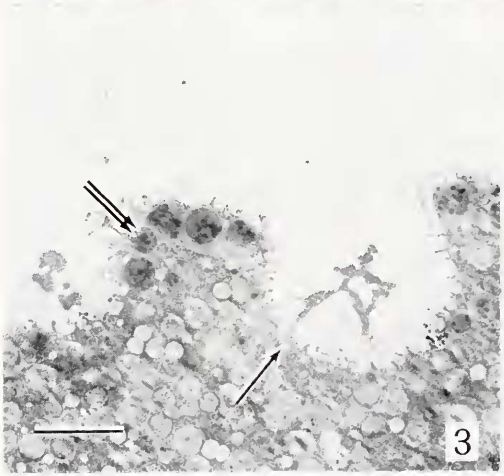
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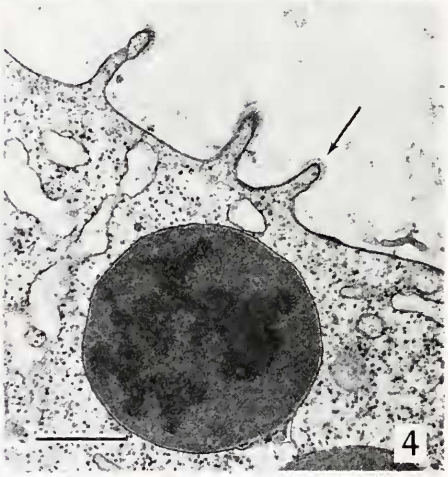
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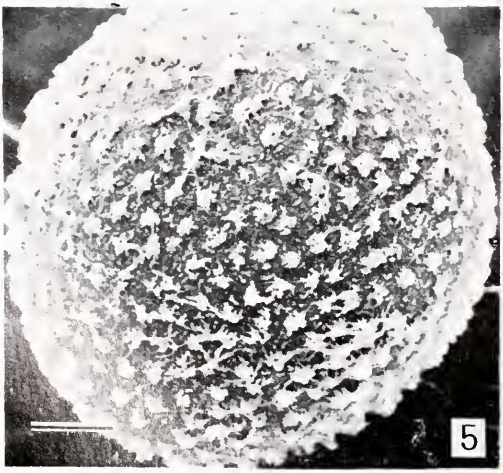
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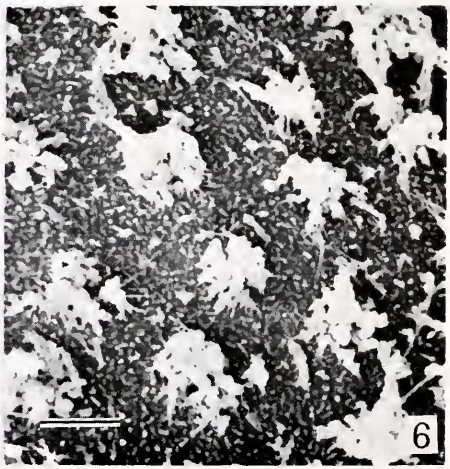
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The exocytosis of a cortical granule at the leading edge of the cortical reaction is shown in Figure 11. Erupted cortical granule material is restrained by and adheres to the inside of the vitelline coat, which is pushed away from the plasma membrane of the egg. As in sea urchins (reviewed by Schmekel, 1975), the fertilization membrane of *Comanthus* is a composite of vitelline coat and cortical granule material. However, in comparison to the fertilization membrane of sea urchins, that of *Comanthus* includes on the order of a hundred times as much cortical granule material.

As exocytosis begins, the cortical granule contents increase in volume and decrease in density (Fig. 11). For several seconds thereafter, the dense patches of the cortical granule material can still be distinguished from the less dense matrix (Figs. 11 and 13). By the time ridge elevation is just beginning, the fertilization membrane has resolved itself into a thin outer component (between the arrows in Fig. 13) and a thick inner component. The outer component has a dense, granular consistency and is roughly 0.1 μ thick; this thickness is nearly ten times that of the vitelline coat, and the outer component presumably includes a large amount of cortical granule material in addition to the vitelline coat. A very thin layer, possibly the vitelline coat, reappears at the outer surface of the outer component 20 min after insemination (Holland and Jespersen, 1973). The inner component of the fertilization membrane, as ridge erection begins, is roughly 2 μ thick and is made up of fibrogranular material derived from the cortical granules (Fig. 13). Microvilli up to 2 μ long are extended from the egg surface as cortical granule exocytosis is taking place; at first, these microvilli project into the substance of the fertilization membrane (Fig. 14).

Several seconds after the start of the cortical reaction, ridges begin to arise on the surface of the fertilization membrane (Fig. 10). These ridges appear between adjacent jelly clumps and form a pattern of hexagons with an occasional pentagon (Figs. 9 and 12). Each polygon of ridges outlines a depression that I will call a facet. Within each facet, a jelly clump remains in close association with the outside of the fertilization membrane (Fig. 14). During the next minute or two, the ridges grow progressively higher, reaching a maximum height of about 15 μ (Figs. 15 and 16). As maximum ridge height is attained, the inner component of the fertilization membrane has become distinctly fibrous (Fig. 17).

The simplest explanation of ridge formation is that the jelly clumps act as physical restraints, holding down the facets, while the ridges are able to rise only in the spaces between adjacent jelly clumps. One can speculate that the vitelline

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FIGURE 1. A dented oocyte with numerous surface pits, each containing a jelly clump (remnants of ovary adhere at top and bottom). The scale line is 30 μ.

FIGURE 2. Surface detail of a dented oocyte with jelly clumps in pits. The scale line is 7 μ.

FIGURE 3. The periphery of a dented oocyte, with pits containing jelly clumps at left and center. The single arrow indicates close association between jelly and the oocyte surface. The twin arrow points to a cortical granule. The scale line is 5 μ.

FIGURE 4. Details at the periphery of a dented oocyte. The arrow indicates the layer of extracellular material capping a microplica. The conspicuous organelle in the cytoplasm is a cortical granule. The scale line is 0.7 μ.

FIGURE 5. An unfertilized egg covered with jelly clumps, which have the same distributional pattern they had on the oocyte. The scale line is 30 μ.

FIGURE 6. Surface detail of an unfertilized egg with jelly clumps. The scale line is 7 μ.

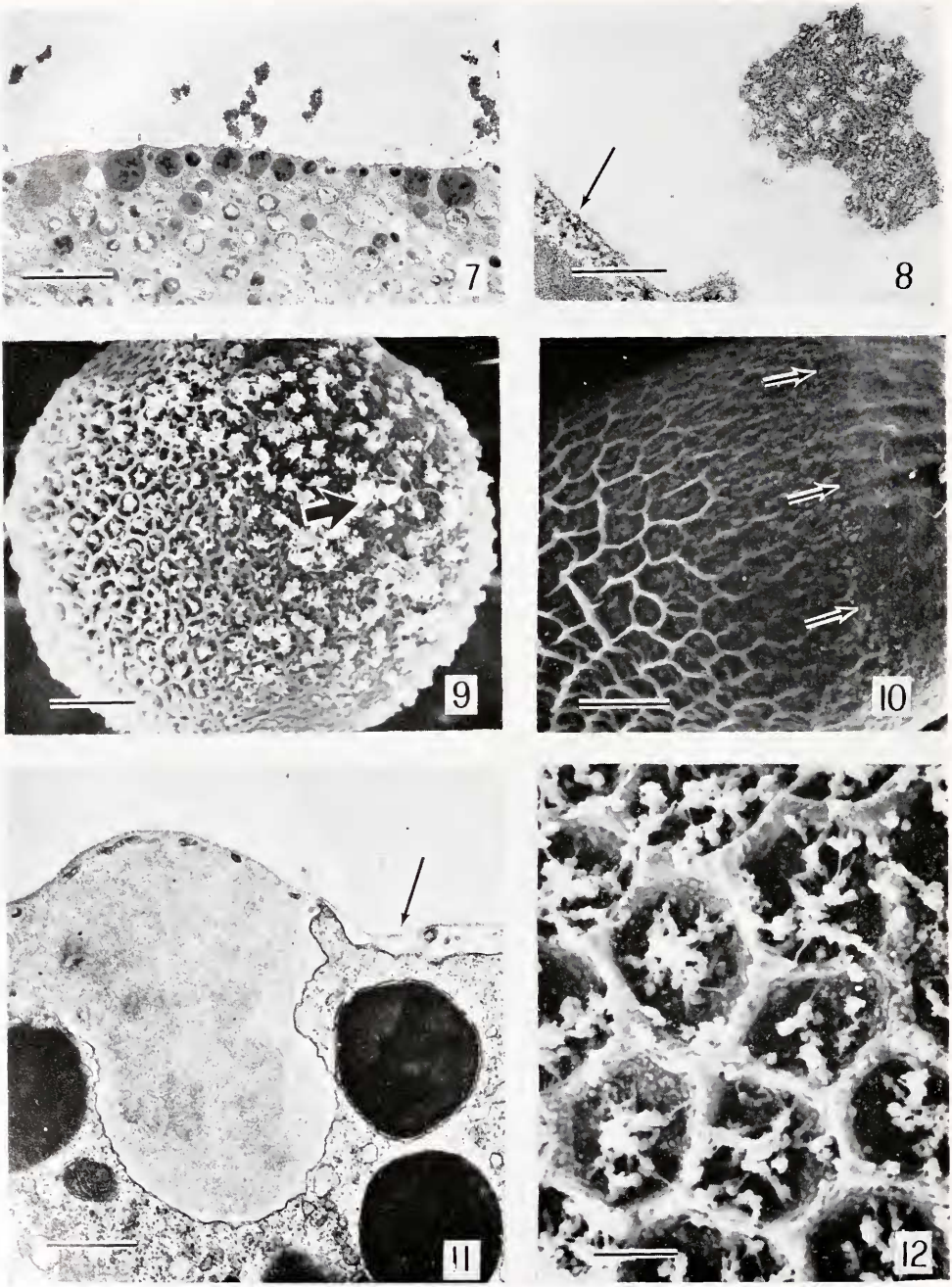


FIGURE 7. The periphery of an unfertilized egg. Portions of jelly clumps appear at left, center and right. Cortical granules are conspicuous just beneath the plasma membrane. The scale line is 5 μ.

coat is elastic enough to permit ridge erection, and that adhesion between each jelly clump and the underlying vitelline coat inhibits lateral spreading of the facet floor. The force causing the ridges to rise is not known with certainty, but could well be osmotic.

As ridge formation is in progress, the inside of the fertilization membrane separates from the underlying plasma membrane of the egg, leaving a perivitelline space several microns wide (Fig. 16, arrow). During fertilization membrane elevation, the microvilli of the egg are pulled out of the substance of the fertilization membrane and are left projecting into the perivitelline space. No trace of a hyaline layer is detectable in the perivitelline space. At 4 min after insemination, jelly clumps are still located in the facets of the fertilization membrane. It is not known whether the jelly ultimately dissolves, since older fertilization membranes were not prepared with alcian blue added to the fixative.

Light microscopic observations of living eggs

If, as the electron microscopy indicates, the presence of jelly clumps on the egg is necessary for ridge formation on the fertilization membrane, then insemination of a jellyless egg should result in production of a ridgeless fertilization membrane. The jelly clumps of the living egg (Fig. 18) can readily be removed by exposure to calcium-free sea water (Fig. 20), a treatment that does not remove the vitelline coat (Fig. 21). At this point there is a technical difficulty, since eggs treated in calcium-free sea water and then inseminated never undergo a cortical reaction, even when returned to normal sea water prior to insemination; the reasons for this failure have not been studied, although lack of jelly could well prevent the sperm from binding to or entering the eggs. Fortunately, eggs treated in calcium-free sea water can undergo a cortical reaction after exposure to ionophore A23187; the reaction occurs whether or not calcium is present in the medium during ionophore administration.

When control eggs, which have not been dejellied in calcium-free sea water, are treated with ionophore A23187, there is a latent period of about 45 sec, and then cortical granule exocytosis occurs simultaneously over the entire surface of the egg. During the 90 sec after exocytosis, a fertilization membrane with surface ridges is

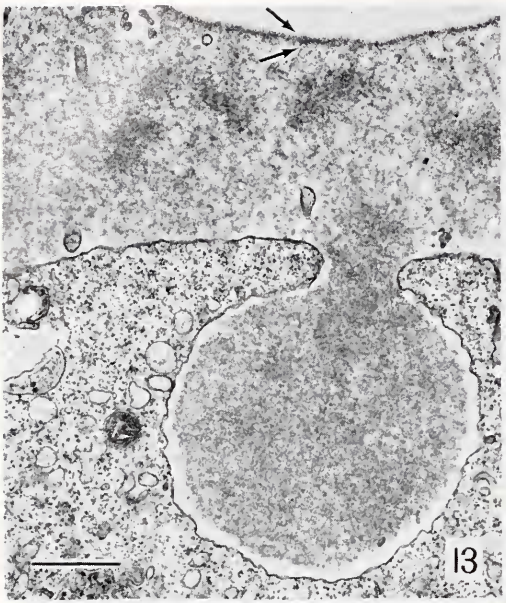
FIGURE 8. Detail at the periphery of an unfertilized egg. The plasma membrane (poorly preserved) is covered with a vitelline coat (arrow). A portion of a jelly clump is at the right. The scale line is 0.5 μ.

FIGURE 9. An egg fixed 70 sec after insemination. The cortical reaction began at left center and, at the instant of fixation, was spreading in the direction of the arrow. At the right, the surface is comparable to that of the unfertilized egg; at the left, the ridges of the fertilization membrane are rising among the jelly clumps. The scale line is 30 μ.

FIGURE 10. An egg fixed 70 sec after insemination with the cortical reaction spreading from left to right. The jelly has been removed to show the leading edge of the cortical reaction (indicated by the arrows). The scale line is 25 μ.

FIGURE 11. Detail of the periphery of an inseminated egg at the leading edge of the cortical reaction, which is proceeding from left to right. The vitelline coat (arrow) restrains the erupting cortical granule material. The scale line is 1 μ.

FIGURE 12. Surface view of the fertilization membrane where the ridges are just beginning to form. The ridges outline polygonal facets in which the jelly clumps are located. The scale line is 7 μ.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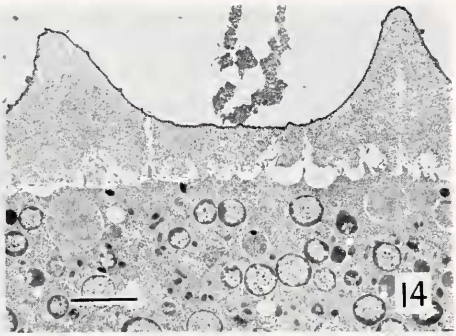
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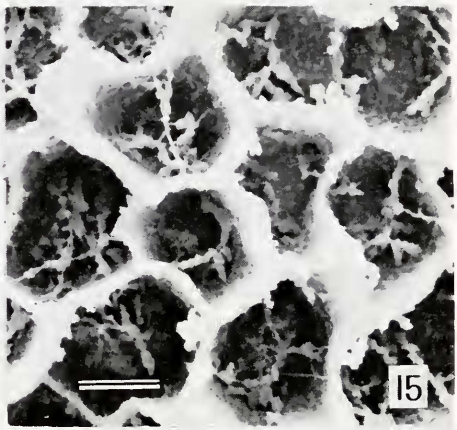
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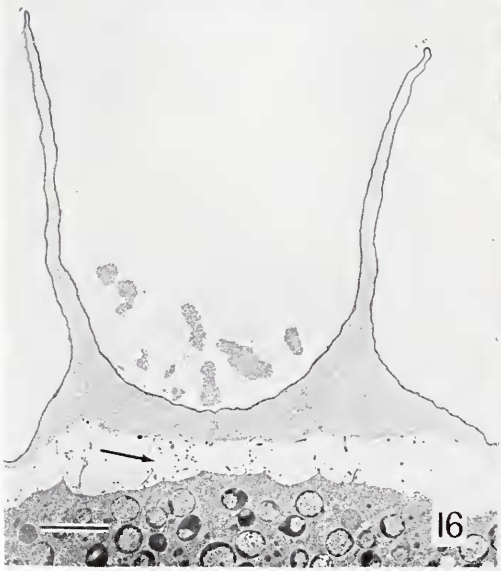
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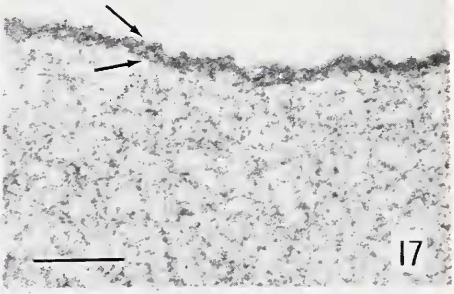
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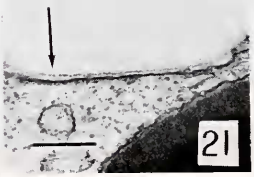
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produced; such a fertilization membrane appears structurally identical to one elicited by insemination both by light microscopy (Fig. 19) and by electron microscopy.

When eggs are de-jellied in calcium-free water for 30 min, returned to normal sea water for 10 min (to prevent excessive swelling of the fertilization membrane) and then activated with ionophore A23187, a ridgless fertilization membrane is produced. This smooth fertilization membrane begins to appear about 45 sec after ionophore administration and, during the next 15 sec, attains maximum thickness (Fig. 22). The production of a smooth fertilization membrane after removal of the jelly strongly supports the argument that the pattern of jelly clumps on the egg surface is a proximate cause of the pattern of ridges on the fertilization membrane surface.

Discussion

The ridge pattern on the fertilization membrane of *Comanthus* can be traced back, *via* the jelly clump pattern on the egg, to a pit pattern on the oocyte surface. How the oocyte initiates and maintains its pitted surface topography remains a mystery. Just when the pits are forming, cytoplasmic fibers temporarily become conspicuous in the oocyte cytoplasm (Holland, Grimmer and Kubota, 1975). It is possible that these fibers could be involved in pit formation; unfortunately, they

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Figure 13. Detail of the periphery of an inseminated egg where the ridges are just beginning to form on the fertilization membrane. The fertilization membrane has resolved itself into a thin, dense outer component (between the arrows) and a thick, less dense inner component. The scale line is 1 μ.

Figure 14. The periphery of an inseminated egg in a region comparable to the far left in Figure 10. A jelly clump (top center) occupies a facet of the fertilization membrane. The scale line is 3 μ.

Figure 15. Surface of the fertilization membrane of an inseminated egg between 1 and 2 min after the passage of the cortical reaction. The surface ridges have attained their maximum height of about 15 μ, and jelly clumps still occupy the facets. The scale line is 7 μ.

Figure 16. The periphery of an inseminated egg between 1 and 2 min after the passage of the cortical reaction. A jelly clump occupies a facet bounded by fully formed ridges. The fertilization membrane has lifted away from the cell surface, leaving a perivitelline space (arrow). The scale line is 3 μ.

Figure 17. Detail of the fertilization membrane between 1 and 2 min after the cortical reaction. The relatively thin, dense outer component (between the arrows) overlies the thick, fibrous inner component. The scale line is 0.3 μ.

Figure 18. A living, unfertilized egg photographed uncompressed under phase contrast (as were the eggs in Figs. 19, 20 and 22). The jelly clumps are conspicuous. The scale line (which is also applicable to Figs. 19, 20 and 22) is 100 μ.

Figure 19. A living egg 2 min after insemination. The fertilization membrane with fully formed ridges is conspicuous. Living eggs 2 min after administration of ionophore A23187 appear exactly the same as this inseminated one.

Figure 20. A living, unfertilized egg 3 min after exposure to calcium-free sea water. The jelly clumps are dissolving and will have vanished completely in a few minutes more.

Figure 21. Detail of the periphery of an unfertilized egg after 30 min in calcium-free sea water. The vitelline coat (arrow) is still present. The scale line is 0.3 μ.

Figure 22. A living egg, de-jellied in calcium-free sea water for 30 min, returned to normal sea water for 10 min and then exposed to ionophore A23187 for 10 min. A ridgeless fertilization membrane has been produced.

have been studied only by light microscopy. The maintenance of the dented surface is not correlated with a conspicuous framework of microtubules of microfilaments in the cortical cytoplasm of the oocyte (Holland, unpublished). Indeed, no microtubules were detected there by TEM (although the same fixation preserved the microtubules of the meiotic spindles), nor were microfilament networks well developed. There is some evidence that cell surface protein may play a role in maintenance of the dented topography of the oocyte, since dented oocytes become smooth 4 min after being placed in sea water containing 0.01% protease (Sigma, type VI from *Streptomyces griseus*) (Holland, unpublished). The protease-smoothed oocytes lose their microplicae, but remain surrounded by jelly clumps. It is not known if the protease acts on the extracellular layer capping the microplicae, on proteins of the plasma membrane, or on both. It is also possible that the protease ultimately has a transmembrane effect on the cortical cytoplasm of the oocyte. Recent work on vertebrate cells *in vitro* also indicates that cell surface protein profoundly influences cell shape (Mallucci and Wells, 1976; Yamada, Yamada and Pastan, 1976).

The jelly clump on the egg consists of tubules approximately 250 Å in diameter. Light microscopic histochemistry indicates that the jelly clumps are rich in nonsulfated acid mucopolysaccharide (Holland, unpublished); therefore, each tubule probably has a polyanionic surface. Such polyanionic tubules could be cross linked with one another by calcium ions, which would explain why the jelly clumps so readily dissolve in calcium-free sea water. The polyanionic nature of the tubules is further indicated by their stabilization when the cationic dye, alcian blue, is added to fixative solutions. The substructure of tightly packed tubules might give the jelly clump as a whole a relatively firm consistency, well suited for holding down the facet floors during fertilization membrane formation. The tubules of the jelly clumps of *Comanthus* appear to differ chemically from the proteinaceous "microtubule mimics" discussed by Wourms and Sheldon (1976, p. 360).

The fertilization membrane of *Comanthus* is probably made up chiefly of proteins derived from the vitelline coat and from structural material originating from the cortical granules. The vitelline coat is soluble in dithiothreitol and is presumably rich in disulfide-linked proteins (Holland, 1976a), while the material derived from the cortical granules gives positive histochemical reactions for protein (Holland, unpublished). At present, nothing is known of the biochemical reactions that bring about the formation of the dense outer component and the fibrous inner component of the fertilization membrane of *Comanthus*.

The maximum height of the ridges is probably held to about 15 μ by one or more of the following: a limited amount of stretch in the vitelline coat, a loss of osmotically active substances from the inner component, or an increase in the structural integrity of the inner component. The long microvilli erected concomitantly with cortical granule exocytosis presumably do not push up the ridges on the fertilization membrane, since these microvilli do not exceed a few microns in length and are as abundant beneath the facets as beneath the forming ridges (Fig. 16). It seems more likely that such microvilli form at exocytosis to help accomodate excess membrane resulting from the sudden fusion of cortical granule membranes with the overlying plasma membrane, a problem discussed by Eddy and Shapiro (1976).

The eggs of *Comanthus* are shed directly into the sea water, which is probably the commonest and most evolutionally primitive mode of reproductive behavior among crinoids (Mortensen, 1937; Holland, 1976c). In crinoids for which reproduction has been studied, the spawning of eggs into the sea water is always followed by the production of an ornamented fertilization membrane (Mortensen, 1920a, 1920b, 1937, 1938; Dan and Dan, 1941). During evolution, crinoids spawning eggs into the sea water are presumed to have given rise to crinoids brooding eggs on or in the body of the mother (Holland, 1976c). The evolution of brooding is associated with the loss of ridges from the fertilization membrane, which is relatively smooth in brooding species (Clark and Clark, 1967, pp. 168 and 636). For an external brooder, ridges on the fertilization membrane would probably be worse than useless, since they would interfere with adherence to the mother's epidermis; for an internal brooder, such ridges would needlessly take up space in the mother's brood pouches.

It should be added that some ophiuroid echinoderms of the genus *Ophiocoma* have ornamented fertilization membranes (Mortensen, 1937), apparently of the type described here for *Comanthus*. The significance of this peculiar coincidence is obscured by the present uncertainty about the phylogenetic relationships among the living echinoderm classes (Ubaghs, 1967).

In the course of normal development, the ultimate fate of the fertilization membrane is dissolution, presumably under the influence of a proteolytic hatching enzyme produced by the embryo (Dan and Dan, 1941). Dissolution begins from the inner surface of the membrane about 13 hr after fertilization, and the embryo hatches out about 2 hr later (Holland, 1976b).

The present investigation is affectionately dedicated to Drs. Jean and Katsuma Dan, who helped show me the way. I am also deeply indebted to Director Hiroshi Terayama and Assistant Director Shonan Amemiya for their kind hospitality and for their generous loan of facilities at the Misaki Marine Biological Station of the University of Tokyo. This work was supported in part by U.S.P.H.S. Biomedical Sciences Support Grant No. R-07011. Ms. Ellen Flentye gave competent assistance with the SEM, and the manuscript was criticized by Dr. David Epel, Ms. Linda Holland, Dr. Mia Tegner and Dr. Victor Vacquier.

Summary

1. This is the first electron microscopic description of fertilization membrane formation in a crinoid echinoderm.

2. The fertilization membrane is a composite structure consisting of a thin vitelline coat plus a thick layer of material originating from the cortical granules.

3. The fertilization membrane elevates from the plasma membrane, leaving a perivitelline space several microns wide; no trace of a hyaline layer appears in this space.

4. By 2 min after the start of cortical granule exocytosis, ridges about 15 μ high are erected on the surface of the fertilization membrane. The ridge pattern is

determined by the egg jelly which is divided into several hundred clumps on the egg surface; each jelly clump apparently acts as a physical restraint on the fertilization membrane surface immediately beneath, and ridges can rise only between the jelly clumps.

5. The morphogenetic role of the egg jelly is corroborated by observations on living eggs; a ridgeless fertilization membrane can be produced if the jelly is first removed from the egg surface.

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